Attachment # 1 GLP Protocol # 3431; Study # GR3249

Gibraltar Laboratories, Inc.
122 Fairfield Road, Fairfield, NJ 07004-2405
Phone: (973) 227-6882 Fax: (973) 227-0812

Current AOAC Edition Hospital Confirmatory Use-Dilution Method [UDM]

1. **Testing Facility**
   Gibraltar Laboratories, Inc.
   16 Montesano Road
   Fairfield, NJ 07004
   Phone: (973) 227-6882
   Fax: (973) 582-1565

2. **Study Director:** Jozef Mastej, Microbiology Manager

3. **Sponsor**
   HSP USA, LLC
   3111 Route 38 Suite 11 # 310
   Mount Laurel, NJ 08054
   Phone: (856) 437-0688
   e-mail: info@hsp-usa.com

4. **Contact Person:** Henry Dao, President and CEO, HSP USA LLC.

5. **Purpose / Objective**
   To determine whether or not the disinfectant kills, in 3-minute, at least 10 of 10 carriers/lot against the test organisms *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

6. **To be submitted to:** EPA [To be "Conducted for": Substantiation of Claims for Hospital disinfectant/hard non-porous surfaces].

7. **References**
   7.1. AOAC Official Method 964.02 Testing Disinfectants against *Pseudomonas aeruginosa* Use-Dilution Method.
   7.2. AOAC Official Method 955.15 Testing Disinfectants against *Staphylococcus aureus* Use-Dilution Method.
   7.3. 40 CFR Section 160, Good Laboratory Practices Regulations.
   7.4. Product Performance Test Guidelines OCSPP 810.2200: Disinfectants for Use on Hard Surfaces - Efficacy Data Recommendations.

8. **Test Material:** Hsp_2O, Active Ingredient: Hypochlorus acid 165 ppm.

9. **Lot Numbers**
   Specify the two lots to be tested for efficacy, as well as manufacturing dates. The Lot #'s will be provided by sponsor on the test date.
   9.1. Lot # 1
   9.2. Lot # 2

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10. **Test System and Justification**
The following organism will be used to support an efficacy claim per the references listed in **Section 7**.
10.1. *Staphylococcus aureus,* ATCC # 6538
10.2. *Pseudomonas aeruginosa,* ATCC # 15442

11. **Media and Equipment**
11.1. AOAC Letheen Broth supplemented with 0.03% Sodium Thiosulfate (neutralization/recovery broth)
11.2. Tryptic Soy Agar (TSA)
11.3. AOAC Synthetic Broth
11.4. Incubator 36 ± 1°C
11.5. Calibrated Timer
11.6. Calibrated Thermometer

12. **Test Conditions**
12.1. Contact Time: 3-minute
12.2. Organic Load: None
12.3. Test Diluent: Ready To Use [RTU]
12.4. Test Dilution: None
12.5. Test Temperature: 20 ± 1.0°C

13. **Testing Grid**

Quantity of carriers to be tested		
Lot #	*Staphylococcus auerus*	*Pseudomonas aeruginosa*
	# carriers	# carriers
1	10	10
2	10	10

14. **Method**
The study will be conducted under EPA GLP regulations 40 CFR Section 160. All of the procedures involved in performance of this study are described in a detailed series of SOPs that are maintained at Gibraltar Laboratories, Inc. SOPs and Logs are referred to in the raw data and are required as part of GLP regulations.

14.1. Test System Identification
All Petri dishes, dilution tubes, etc. will be labeled with the following information: name of the organism, date and GBL number.

14.2. Carrier Preparation: The 8 ± 1 mm o.d. x 6 ± 1 mm i.d. x 10 ± 1 mm length stainless steel penicylinders will be biologically screened by soaking overnight (approximately 12 hours) in 1N NaOH and rinsed several times with tap water. Collect a portion of the last rinse water and add 2-3 drops of 1% phenolphthalein; if any NaOH remains, the phenolphthalein turns pink, indicating the need for additional rinsing. Continue to rinse the carriers until the addition of phenolphthalein does not produce a color change, and then rinse twice more with H_2O. Placed cleaned carriers in a vessel with closures, cover with deionized water and sterilize, cool and hold at room temperature up to 3 months.

14.3. Test Culture Medium: AOAC Synthetic Broth for preparation of organisms. Before using for daily transfers of test cultures, aseptically add 0.1 mL sterile 10% glucose (dextrose) solution per tube. Grow

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cultures with tube slanted.

14.4. Recovery Media: AOAC Letheen Broth supplemented with 0.03% Sodium Thiosulfate; 10 mL in a 20 x 150 mm test tube for subculture recovery from medicated carrier.

14.5. Culture Preparation of *Staphylococcus aureus*

14.5.1. Defrost a single cryovial of *Staphylococcus aureus* at room temperature and briefly vortex to mix. Add 10 µL of the thawed frozen stock to a tube containing 10 mL synthetic broth, vortex, and incubate at 36 ± 1°C for 24 ± 2 hours. Only one daily transfer is required prior to initiation of the final test culture. Daily cultures may be subcultured for up to 5 days; each daily may be used to generate a test culture.

14.5.2. For this final subculture step, inoculate a sufficient number of 25 x 150 mm tubes containing 10 mL synthetic broth with 10 µL per tube of the 24 hours synthetic broth culture and incubate at 36 ± 1°C for 48 to 54 hours. Vortex, mix synthetic broth test cultures 3-4 seconds and let stand 10 minutes at room temperature before continuing. Remove the upper portion of each culture, leaving behind any debris or clumps, and transfer to a sterile vessel: pool cultures in the vessel and swirl to mix. Aliquot 24 mL portions into sterile 25 x 150 mm.

14.6. Culture Preparation of *Pseudomonas aeruginosa*

14.6.1. Defrost a single cryovial of *Pseudomonas aeruginosa* at room temperature and briefly vortex to mix. Add 10 µL of the thawed frozen stock to a tube containing 10 mL synthetic broth, vortex, and incubate at 36 ± 1°C for 24 ± 2 hours. Only one daily transfer is required prior to initiation of the final test culture. Daily cultures may be subcultured for up to 5 days; each daily may be used to generate a test culture.

14.6.2. For this final subculture step, inoculate a sufficient number of 25 x 150 mm tubes containing 10 mL synthetic broth with 10 µL per tube of the 24 hours synthetic broth culture and incubate at 36 ± 1°C for 48 to 54 hours. Do not vortex or shake 48 to 54 hours test culture. The pellicle from the 48 to 54 hours cultures will be removed from the broth before mixing by gently aspirating the pellicle off the surface of the growth media with a Pasteur pipet attached to a vacuum apparatus. Any distribution of the pellicle resulting in dropping, or breaking up of the pellicle in the culture before or during its removal renders the culture unusable in the use-dilution test. This is extremely critical because any pellicle fragment remaining will result in uneven clumping and layering of organism on the cylinders, allowing unfair exposure to disinfectant and causing false positive results. Vortex, mix synthetic broth test cultures 3-4 seconds and let stand 10 minutes at room temperature before continuing. Remove the upper portion of each culture, leaving behind any debris or clumps, and transfer to a sterile vessel: pool cultures in the vessel and swirl to mix. Aliquot 24 mL portions into sterile 25 x 150 mm.

14.7. Carrier Inoculation: Using a sterile hook, aseptically transfer 24 carriers (20 test, 1 viability control, 2 quantitative controls, 1 extra) repeat to achieve requisite number of carriers prepared as in section **14.2** into each of the tubes containing the test culture. Drain the water from the carriers by tapping them against the side of the tube before transferring to the culture. The test culture must completely cover the carriers. Multiple carriers may be transferred on a single wire hook.

14.7.1. Alternatively, the water may be siphoned off the carriers and the 24 mL test culture added directly to the carriers without transferring.

14.7.2. A larger vessel (e.g., 250 mL beaker) containing up to 100 carriers may also be substituted for 25 x 150 mm test tube for carrier inoculation step; however, the ratio of one carrier to 1 mL culture must

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Phone: (973) 227-6882 Fax: (973) 227-0812

be maintained.

The test culture must completely cover the carriers. If a carrier is not covered, gently shake the tube, or reposition the carrier within the tube with a sterile wire hook. Be sure to inoculate a sufficient number of carriers for the test. The carriers will be soaked for 15 ± 2 minutes in the respective test system culture broth ~~containing 5% organic load~~. Carriers will then be transferred and allowed to dry on Whatman 2 filter paper in a sterile petri dish at 36 ± 1°C for 40 ± 2 minutes. Place no more than 12 carriers in a Petri dish.

14.8. Disinfection: Each contaminated and dried carrier will be placed into a separate 25 x 150 mm test tube containing 10 mL of the disinfectant solution for ~~2~~ 3 minute at 30 second staggered intervals in a 20 ± 1°C water bath. * error form correction 3 minutes JMC 4/9/15

14.9. Subculture: Each medicated carrier will then be transferred by a sterile wire hook at 30 second staggered intervals to 10 mL of neutralization/recovery broth (See Section **11.1**).

14.10. Incubation: The neutralization/recovery broth tubes will be incubated for 48 ± 2 hours at 36 ± 1°C. The neutralization/recovery broth tubes will be visually observed for gross turbidity and for typical growth.

14.11. Verification of positive carriers: In the event of a positive result the character of the organism will be compared to the test system so as to rule out the possibility of a false positive or otherwise variant result. The extent of verification testing will be as follows:
Verification tests will be done on all tubes if there are <5 positives and 20% of the tubes if there are more than 5 positives. These positive carriers will all be examined for the test organism by inoculating onto TSA and selective media. The TSA and selective media plates will be incubated for 18 to 24 hours at 36 ± 1°C. These plates will be examined for colonial morphology characteristic of the test organism and gram stain. If required to confirm the identity of the test system the Sponsor will be contacted to authorize identification by Vitek Senior Microbial Identification System on representative colonies.

14.12. Neutralization Confirmation: A neutralization confirmation procedure must demonstrate the recovery of a low level (10 to 100 cfu) of the test organism in the neutralization/recovery jar. Typical growth in jars confirms effective neutralization.
Additional 3 carriers will be disinfected as per section **14.8**. Dilute a 24 to 48 hours culture of the test organism in sterile saline to achieve 100 to 1000 cfu/mL. Add 0.1 mL diluted suspension to each tube to delivery 10 to 100 cfu per tube. The inoculated test tubes will be incubated for 48 ± 2 hours at 36 ± 1°C and observed for turbidity. Results will be recorded as + for growth and 0 for no growth. Confirm the neutralization inoculum (e.g. number of bacteria in the 0.1 mL diluted suspension used for inoculation) by duplicate pour plating 0.1 mL diluted suspension/plate. The plates will be poured with TSA and incubated for 48 ± 2 hours at 36 ± 1°C. Count the colonies on plates to determine inoculum challenge.

14.13. Viability Controls: This control will verify that typical growth occurs and that a pure culture was used. After the drying of the inoculated carriers (from **14.7**) three carriers will be directly transferred into neutralization/recovery broth (no disinfection treatment) and incubated for 48 ± 2 hours at 36°C ± 1°C. Positive growth in each tube validates test system viability

14.14. Enumeration of viable bacteria from carriers (Carrier Counts): This control will verify that between 1.0 x 10^6 to 1.0 x 10^7 cfu was present on each carrier (Log_{10} 6.0 to Log_{10} 7.0). After inoculated carriers have dried, randomly select one carrier from each petri dish (e.g. 6 petri dishes with 12 carriers per dish).

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Carriers should be assayed in two sets of ~~three~~ two *, one set immediately prior to conducting the efficacy test and one set immediately following the test. Place each individual carrier in a tube 10 mL of neutralization/recovery broth. The carriers will be sonicated for 1-minute ± 5-second in the neutralization/recovery broth to dislodge the adhering organisms. This will be diluted to 10^{-5}, and then 1.0 mL aliquots in duplicate from each dilution will be plated using Trypticase Soy Agar (TSA) and incubated at 36°C ± 1°C for up to 48 ± 2 hours. The colony counts will be counted and extrapolated to cfu per carrier. Calculate the $\log_{10}$ density (LD) for each carrier by taking the $\log_{10}$ of the density (per carrier). The mean LD across carriers is the mean TestLD for the test. The mean TestLD must be at least 6.0 (1.0×10^6) and not above7.0 (1.0×10^7). * error, some correction JM 4/9/15

14.15. Retesting Guidance:

14.15.1. For tests where the product passes and the mean TestLD value is above 7.0, no retesting is necessary.

14.15.2. For tests where the product fails and the mean TestLD is below 6.0, no retesting is necessary.

14.15.3. For tests where the product fails and the mean TestLD is above 7.0, retesting may be conducted.

14.16. Sterility Controls: These controls will verify that the media was sterile and aseptic technique during carrier transfer process was used.

14.16.1. Agar Control

Two sterile Petri dishes will be poured with sterile TSA from each lot of media used in the test and will be incubated along with the test sample test tubes.

14.16.2. Recovery Broth Control

Two unopened neutralization/recovery broth test tubes from the same lots used in the test will be incubated along with the test sample test tubes.

14.16.3. Recovery Broth with carriers

To verify the sterility of the stainless steel penicylinders [carriers], as above, except that one stainless steel penicylinders will be added to neutralization/recovery broth test tube at start of samples test (prior to first tube) and second after all test carriers (last tube) have been transferred to neutralization/recovery broth and will be incubated along with the test samples.

These controls will verify that the media, bovine serum, stainless steel penicylinders were sterile and aseptic technique during carrier transfer process was used.

15. **Randomization:** Not Applicable

16. **Storage Facilities:** Test material will be stored at controlled room temperature conditions unless refrigeration or freezing is required.

17. **Stability and Purity:** The Sponsor is responsible for chemical stability and purity information.

18. **Hazards:** The sponsor is to notify GBL of any potential hazard; ordinary precautions in handling test material will be taken unless otherwise specified. It is the responsibility of the sponsor to take back all test material after testing.

19. **Protocol Changes:** If it becomes necessary to change the approved protocol after the study has been initiated, a protocol amendment will be prepared between the Study Director or designee and the Sponsor. As soon as practical, this change and the reasons for it will be signed by the Study Director or designee and the Sponsor. This document will then be attached to the protocol as an addendum. If, in the opinion of the

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Study Director, it is necessary to abort a test and perform a retest, a single final report will be issued pertaining to the aborted test and the retest. A protocol amendment will not be issued. In this circumstance it is likely that the proposed study initiation and termination dates will be different than specified in the protocol.

20. **Report:** The report will include the sample number and reference, the description of the test material, how the study was conducted, the dates the testing was initiated and terminated. This report will conform to all requirements outlined in 40 CFR Section 160, Good Laboratory Practices Regulations. Sponsor will be provided with a draft report for review prior to finalization.

21. **Archives:** 122 Fairfield Road, Fairfield, New Jersey 07004-2405.

22. **Records to be maintained:** The final report of this study as well as all raw data accumulated during the study will be kept in the archives of Gibraltar Laboratories, Inc. for a period of at least 10 years, unless notified by sponsor in writing, after which the documents will be returned to the sponsor.

23. **Statistical Method:** None. Basic arithmetic will be used.

24. **Scheduling and Disclaimer of Warranty**
Proposed Initiation Date* To be specified on the acknowledgement notification
Proposed Completion Date* To be specified on the acknowledgement notification
* Indicates "proposed" dates; does not guarantee testing can begin (or end) on these dates. Sponsor will be kept apprised of status of testing. If not completed, dates will be specified in final written report.

The EPA and FDA make no predications, guarantees or warranties in respect to either the outcome of the test or the approval of the data

If a, test or portion of it has to be repeated, because of a technical or procedural problem, as determined by GBL, it will be done without charge.

If a sponsor requests a repeat test, they will be billed for an additional test.
Neither the names of GBL nor any of its personnel are to be used for advertisement purposes without written permission.

The data supplied will indicate if the specified test organisms can be inactivated on a hard surface. The data is not to be used to represent that product will prevent, treat or cure infections caused by these bacteria.

Sponsor is responsible for any rejection by the EPA or FDA of their submission concerning style, format, collation, pagination, etc. To prevent this, Sponsor should carefully review GBL report and notify GBL of any perceived deficiencies in these areas before submitting the GBL report to the EPA or FDA. GBL will make such corrections, as Sponsor feels are necessary, without altering the technical data.

25. **Limitations:** No representations are made that the EPA or FDA will accept without comment, criticism, rejection or further inquiry the data in the GBL report. There will be a charge for any additional work performed in excess of the data submitted in the final report unless as cited in 23.

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26. **Disposition of Test Material:** After all studies are completed, unless otherwise directed, the unused test material, if any, will be discarded or destroyed in accordance with GBL policy and State and Federal regulations.

27. **Disposition of Final Report:** Sponsor must provide GBL with an official copy of the GBL final report submitted to the FDA, EPA, or other regulatory agencies.

28. **Certificate of Analysis:** Sponsor must provide Gibraltar Laboratories with a certificate of analysis on the active ingredient or request Gibraltar Laboratories to determine it at extra cost.
[X] Certificate of Analysis enclosed
[ ] GBL will perform an analysis of the potency of each lot submitted prior to initiating microbiological testing.

29. **Safety Information**
(Please attach MSDS sheets or other information and special precautions if necessary).
Sponsor assumes liability for worker injury if available precautions and warnings are not supplied. Please check below where appropriate and attach MSDS.
[ ] No precautions necessary (neither toxic nor irritating)
[ ] Skin irritant (Refer to MSDS) [ ] Inhalation toxicity
[ ] Eye irritant (Refer to MSDS) [ ] Other toxicity
[ ] Special precaution for lab worker See enclosed MSDS

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Protocol Approval Form

Protocol Title: Current AOAC Edition Hospital Confirmatory Use-Dilution Method [UDM]

Date Written: April 02, 2015

Protocol Number: 3431
Study Number: GR3249

Protocol Approved By

Gibraltar Laboratories, Inc.
Study Director
Jozef Mastej, Microbiology Manager

4/8/2015
Date

Protocol Approved By

HSP USA, LLC
Study Sponsor
Henry Dao, President and CEO, HSP USA LLC.

4/3/15
Date

GIBRALTAR
LABORATORIES

122 FAIRFIELD RD., FAIRFIELD, NJ 07004-2405 16 MONTESANO RD., FAIRFIELD, NJ 07004-2405 *
PHONE: [973] 227-6882 * FAX: [973] 227-0812
e-mail: info@gibraltarlabsinc.com * internet: www.gibraltarlabsinc.com

Attachment # 2 Certificate of Analysis Lot # 150301

Study Title

Characterization Assay of HSP_2O

Study Director

Charles Willis

Final Report Date

April 1, 2015

Testing Facility

Case Laboratories, Inc.
622 Route Ten
Whippany, NJ 07981
Tel. 973-428-9666
Fax 973-887-4419

Sponsor

HSP USA LLC
3111 Route 38, Suite 11, #310
Mount Laurel, NJ 08054
Tel. 856-437-0688
Fax 866-779-8079

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LABORATORIES

122 FAIRFIELD RD., FAIRFIELD, NJ 07004-2405 16 MONTESANO RD., FAIRFIELD, NJ 07004-2405 *
PHONE: [973] 227-6882 * FAX: [973] 227-0812
e-mail. info@gibraltarlabsinc.com * internet: www.gibraltarlabsinc.com

Attachment # 2 Certificate of Analysis Lot # 150301 – continued

Certificate of Analysis

Name:	HSP_20
Lot Number:	150301
Hypochlorous Acid Target Concentration:	165 ppm
Date of Analysis:	03/25/15

Result

Test Method	**Hypochlorous Acid**
ASTM D2022-89	150 ppm

Charles Willis
Study Director
Case Laboratories, Inc.

04/01/2015
Date

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122 FAIRFIELD RD., FAIRFIELD, NJ 07004-2405 16 MONTESANO RD., FAIRFIELD, NJ 07004-2405 *
PHONE: [973] 227-6882 * FAX: [973] 227-0812
e-mail: info@gibraltarlabsinc.com * internet: www.gibraltarlabsinc.com

Attachment # 3 Certificate of Analysis Lot # 150302

Study Title

Characterization Assay of HSP_2O

Study Director

Charles Willis

Final Report Date

April 1, 2015

Testing Facility

Case Laboratories, Inc.
622 Route Ten
Whippany, NJ 07981
Tel. 973-428-9666
Fax 973-887-4419

Sponsor

HSP USA LLC
3111 Route 38, Suite 11, #310
Mount Laurel, NJ 08054
Tel. 856-437-0688
Fax 866-779-8079

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122 FAIRFIELD RD., FAIRFIELD, NJ 07004-2405 16 MONTESANO RD., FAIRFIELD, NJ 07004-2405 *
PHONE: [973] 227-6882 * FAX: [973] 227-0812
e-mail: info@gibraltarlabsinc.com * internet: www.gibraltarlabsinc.com

Attachment # 3 Certificate of Analysis Lot # 150302 – continued

Certificate of Analysis

Name:	HSP_20
Lot Number:	150302
Hypochlorous Acid Target Concentration:	165 ppm
Date of Analysis:	03/25/15

Result

Test Method	**Hypochlorous Acid**
ASTM D2022-89	149 ppm

Charles Willis
Study Director
Case Laboratories, Inc.

04/01/2015
Date

Study No. 8288
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